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Scotin: A new p63 target gene expressed during epidermal differentiation

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Abstract

p63, a member of the p53 family, is transcribed from two different promoters giving rise to two different proteins: TAp63 that contains the N-terminal transactivation domain and ΔN that lacks this domain. In this article we describe a new target gene *Scotin* induced by TAp63 during epithelial differentiation. This gene was previously isolated as a p53-inducible proapoptotic gene and the protein is located in the endoplasmic reticulum and in the nuclear membrane. Scotin expression is induced in response to endoplasmic reticulum (ER) stress in a p53 dependent or independent manner. We detected Scotin upregulation in primary keratinocyte cell lines committed to differentiate. In this paper we also show that Scotin is expressed in the supra basal layer of the epidermis in parallel with TAp63, but not ΔNp63 expression. We conclude that Scotin is a new p63 target gene induced during epithelial differentiation, a complex process that also involves ER stress induction.

Keywords: Scotin; p63; ER stress; GADD153; Epithelial differentiation

The transcription factor p63, belonging to the p53 gene family [1], is transcribed from two different promoters, generating two classes of proteins: one containing an N-terminal transactivation domain (TAp63) and another that lacks this domain (ΔN) [2–4] In addition, alternative splicing at the 3′ end of the transcripts (TAp63 or ΔNp63) generates three different C-terminal splicing variants: α,β,γ. ΔNp63 can act as dominant-negative suppressor of the TA isoforms [5] and is also endowed with its own transcription-activating function [6–8]. During the last few years, evidence has accumulated on the important role of p63 in the epidermal differentiation program. Mice lacking *p63* show severe limb, craniofacial, and skin defects and die soon after birth [9–12]. Genetic complementation of *p63*−/− mice with TAp63α, ΔNp63α or both suggest that ΔNp63 is important for maintaining the proliferative potential of the basal layer, whereas TAp63 contributes by acting synergistically and/or subsequently to ΔNp63 to allow differentiation [13]. Indeed, TAp63 is capable of inducing growth differentiation factor 15 (GDF15) expression after it has been recruited to the proximal p53/p63 binding site located on the *GDF15* promoter and this factor plays a critical role in the regulation of keratinocyte differentiation. [14]. Little is known about the p63, and especially TAp63, target genes relevant for epidermal formation and differentiation, but Notch signaling seems to be important for the commitment of embryonic keratinocytes to terminal differentiation [15]. p63 acts as a selective modulator of Notch 1-dependent transcription and also transactivates the Notch ligand, JAGG-1 [16,17].

In this paper we demonstrate that during epidermal differentiation p63 is capable of inducing *Scotin* gene

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expression. This gene was previously identified as a p53-inducible proapoptotic gene located in the endoplasmic reticulum (ER) and in the nuclear membrane. Scotin can induce apoptosis, independently of p53, in response to ER stresses [18]. Endoplasmic reticulum stress can be triggered by a number of changes in normal ER function (accumulation of unfolded, misfolded or excessive protein, ER lipid or glycolipid imbalances etc.) and as a consequence of impaired ER function, luminal calcium is released from the ER stores and apoptotic signaling is triggered [19]. Our laboratory previously demonstrated that TAp73α, another member of the p53 family, is capable of inducing *Scotin* and ER stress [20,21]. TAp73α was also able to induce GADD153/CHOP, a bZIP transcription factor, induced under ER stress conditions [22–24]. GADD153 is implicated in programmed cell death induced by ER stresses and is upregulated during the differentiation of normal mammary epithelial cells [25].

In this work we have investigated the correlation between *Scotin* induction, mediated by increased TAp63 expression in the upper layers of the differentiating epidermis, during the complex process that leads to formation of the cornified envelope.

Materials and methods

RTqPCR. Real-time PCR was performed on an ABI-7500 SDS instrument (Applied Biosystem, Foster City, CA, USA) using the Platinum SYBR Green qPCR SuperMix UDG without ROX (Invitrogen Life Technologies, Carlsbad, CA n°cat: 11733–046) in a total volume of 25 μl. The PCR reactions were performed using the following primers: hScotF1 (+) (5′-TTG GAG GCT GAG GAT AAG GGG-) hScotR1 (−) (5′-TGT GGA GCG AGG AAA GGT GTG-) hGADD153F (+) (5′-GCT TCT CTG GCT TGG CTG ACT-) hGADD153R (−) (5′-CCA GGG AGC TCT GAC TGG AA-), hActinF (+) (5′-AAA GAC CTG TAC GCC AAC A-) h ActinR (−) (5′-CGG AGT ACT TGC GCT CAG-) hTAp63F (+) (5′-GGA CTG TAT CCG CAT GCA G) hTAp63R (+) (GAG CTG GGC TGT GCG TAG), hDNp63F (GAA GAA AGG ACA GCA GCA TTG AT), hDNp63R (−) (GGG ACT GGT GGA CGA GGA G). The reaction was performed using the following PCR program: 50 °C for 2 min 95 °C for 3 min followed by 40 cycles of 94 °C for 20 ″ and 59 °C for 40″.

Primary keratinocytes, cell cultures, and imaging. Primary keratinocytes were isolated according to Yuspa et al. (1989) from the skin of new born mice (CD-1 mouse strain). The skin was floated overnight in trypsin/EDTA at 4 °C and primary keratinocytes were isolated. Cells were cultured on collagen-coated dishes in medium supplemented with 0.05 mM Ca^{2+}, while 1.2 mM Ca^{2+} was used to differentiate primary keratinocytes. TAp63α and ΔNp63α-inducible Saos-2 cells were cultured as described by [26]. HaCat cells were maintained in DMEM with 10% FBS. Embryos were fixed as described by [13]. The following primary antibodies were used: monoclonal anti-p63 (Ab4, Neomarkers, Fremont, California, USA; 1/300), rabbit polyclonal anti-mouse Scotin (JC105; dilution 1/150).

Cell cultures (HaCat and Saos-2) were plated on 20 mm glass coverslips at a density of 30,000 cells/cm^2 or 25,000 cells/cm^2. After 24 h Saos-2 transfected cells or HaCat cells, committed to differentiate (1.2 mM Ca^{2+} and 0.1% FBS), were fixed as described in Gressner et al. (2005). Cells were then incubated for 1 h at RT with the following antibodies: monoclonal anti-GADD153 (Clone B3, Santa Cruz; 1/100 dilution), and polyclonal anti-loricrin (Covance; 1/200 dilution). Fluorescence was evaluated by confocal microscopy (Nikon, C1 on Eclipse TE200; EZC1 software).

Transfection and Western blots. Cells were transiently transfected (Lipofectamine 2000 Invitrogen, Life Technologies, Carlsbad, CA) and Western blots were performed as described by Candi et al. (2006) using the following primary antibodies: monoclonal anti-p63 (Ab4 clone 1/500), polyclonal anti-tubulin (H-235, Santa Cruz, 1:1000 dilution) polyclonal anti-K10 (Covance; 1/500 dilution), polyclonal anti-mouse Scotin (JC105; 1:1000 dilution), polyclonal anti-human Scotin (H105; dilution 1/1000) and polyclonal Involucrin (Covance; 1:1000 dilution). Proteins were detected using the ECL method.

Results

Since p53 and TAp73α are able to induce *Scotin* expression and endoplasmic reticulum (ER) stress [18,20], we evaluated the presence of ER stress during the epidermal differentiation program in relation to the ability of *p63* to activate *Scotin* expression. To investigate the ability of p63 to trigger Scotin expression, we performed RTqPCR experiments (Fig. 1A) using Tet-On cell lines which can be induced to express TAp63α, ΔNp63α or p53 (positive control) in the absence of interfering endogenous p53. Saos-2 cells are p53 null and the expression of endogenous p63 is only weakly detectable with RT-PCR techniques. This allows complete exclusion of the contribution of p53 and minimizes the effect of endogenous p63 expression. The results confirmed the ability of p53 [18] and TAp63α to induce *Scotin* expression. ΔNp63α does not induce Scotin expression; rather ΔNp63α appears to reduce endogenous *Scotin* mRNA (Fig. 1A). This data has been confirmed by immunofluorescence on TAp63α transfected Saos-2 which shows Scotin expression only in cells where expression of TAp63 is clearly detectable (e.g. transfected; Fig. 1B). The induced Scotin appears as aggregates in the ER indicating the presence of ER stress, as previously shown [18,20]. These aggregates could be detected following TAp63α expression but not after the induction of ΔNp63α (data not shown).

Since ER stress can be confirmed by the evaluation of the presence of the GADD153 transcription factor we tested GADD153 expression in the epithelial HaCat cell line, which expresses endogenous p63. HaCat cells were induced to differentiate, using keratin 10 (expressed in the epidermal suprabasal layer) as a marker of differentiation (Fig. 2A). RTqPCR has been used to demonstrate that the induction of *TAp63α* expression (Fig. 2B) is accompanied by the induction of *Scotin* and *GADD153* transcripts during the differentiation program. The former gene is upregulated by more than 50% (Fig. 2C) while expression of the latter is more than doubled (Fig. 2D). The Supplementary Fig. 1B clearly demonstrates also that in differentiating HaCat cell line, there is a nuclear relocalization of GADD153. Another marker of terminal differentiation, loricrin, was used to confirm the differentiation of the HaCat cells (S1, lower panels).

To further investigate the importance of Scotin during epidermal differentiation we analyzed its expression in the mouse. To facilitate the analysis of Scotin expression, the skin of new born mice has been used, since it is hairless although the epidermis is fully differentiated. The results demonstrate that Scotin is expressed in the upper layers